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**FABRICATION OF BIOLOGICAL MICROARRAYS USING
MICROCANTILEVERS**

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Abstract :

Arrays of silicon-based microcantilevers with properly designed passivated aluminum electrodes have been used to generate microarrays by depositing microspots of biological samples using a direct contact deposition technique. The approach proposed here can be compared to the dip-pen technique but with the noticeable difference that electrostatic fields are generated onto the cantilevers to increase the height of liquid rise on the cantilever surface when dipping them into the liquid to be deposited. Both electrowetting through the reduction of the contact angle and dielectrophoresis through electrostatic forces can be used to favor the loading efficiency. These phenomena are particularly pronounced on the microscale due to the fact that physical scaling laws favor electrostatic forces. Moreover, at this scale, conductive heat dissipation is enhanced and therefore Joule heating can be minimized. Using this approach, with a single loading, arrays of more than a hundred spots, from the femtoliter to the picoliter range, containing fluorescent-labelled oligonucleotides and proteins were directly patterned on a glass slide.

Microarray assays are a major breakthrough which has revolutionized biomedical industry. Indeed, they made possible the fusion between genome research and drug discovery which in turn has provided novel, highly accurate detection methods, faster analytic processes, and rapid gene expression monitoring.

All technologies used to manufacture microarrays include photolithography,¹ ink-jetting,² mechanical microspotting³⁻⁴ and derivatives thereof. Photolithographic DNA synthesis allows production of high-density oligonucleotides microarrays in terms of the number of spots deposited per square centimeter. To produce microarrays with low and medium densities, two main alternatives based on delivering pre-synthesized oligonucleotides have been developed : mechanical microspotting using a direct contact between the deposition tool and the surface and a non-contact method such as ink-jetting.

Recently, the demand for massive production, lower costs and improved performance in terms of speed and sensitivity, has led to the development of promising approaches based mainly on micro and nanotechnologies.⁵⁻⁶

State-of-the-art ink-jet printing techniques allows minimum feature sizes in the micrometer scale and examples in the literature have demonstrated the compatibility of such an approach with silicon-based micro and nanotechnologies for the manufacture of high-quality microarrays.⁷ To reach the sub-femtoliter scale in terms of the volume delivered on a flat surface, that is, to deliver spots with submicrometer sizes, the contact method seems to be the most adequate. In this respect, it has recently been demonstrated that oligonucleotide and protein nanoarrays with submicrometer features could be generated using the dip-pen nanolithography (DPN).⁸⁻⁹

In this letter, a microcantilever-based dispensing technique is presented to deposit picoliter biological samples using a contact method.

The approach relies on an improved dip-pen lithography not in terms of spot size (our microarrays were fabricated with micrometer features whereas with the DPN one could reach the submicrometer scale⁹), though it could easily be decreased using similar cantilevers with a sharp tip, but in terms of liquid loading efficiency. Indeed, passivated aluminum electrodes properly designed and located along the channels and reservoirs on the cantilevers permit an active control of the height of the liquid rise on the cantilevers that improves the loading efficiency when they are immersed into the biological solutions to be deposited. This approach is also particularly interesting since these cantilevers can easily be cleaned and therefore dipped into different solutions without significant cross contamination. This also circumvents the need for replacing the cantilevers for depositing various solutions as is the case with the dip-pen nanolithography¹⁰ and makes this technique really efficient for generating high-density biological microarrays. Another salient feature is that microcantilever arrays are used in parallel, thereby enhancing throughput and speed.

The technological process developed for fabricating the microcantilevers is based on conventional microfabrication techniques. Wafers are 100 mm phosphorus-doped (100) Silicon-On-Insulator (SOI) substrates with a 5- μm -thick top Si layer. A low temperature silicon dioxide (LTO) is deposited by Low Pressure Chemical Vapor Deposition (LPCVD) and a lift-off process is used to define the aluminum electrodes. Electrode passivation is obtained by depositing a second low temperature silicon dioxide layer by LPCVD. The second part of the process consists in patterning and releasing cantilevers by Deep Reactive Ion Etching (DRIE). A Reactive Ion Etching (RIE) is then performed for the silicon dioxide and a second one for the top Si layer to pattern the microcantilevers. During this operation, microchannels and microreservoirs are etched through within the microcantilevers. Finally, a DRIE is carried out on the backside. Etching is stopped by the interfacial oxide layer of the

SOI substrate. This silicon dioxide layer is then etched from the backside by RIE to release the cantilevers.

Arrays of cantilevers (2 mm long, 210 μm wide, 5 μm thick) with a 450 μm spacing were fabricated. Figure 1 shows an array of four cantilevers with passivated aluminium electrodes located along 5- μm -wide channels and reservoirs. These arrays are then used within the framework of a computer-controlled three-axis translation stage supplied by Sigma Koki that allows precise positioning of the cantilevers relative to the surface.

During the experimental procedure, an electrostatic field is used to improve the loading efficiency of the microchannels. Both electrowetting through the reduction of the contact angle¹¹ and dielectrophoresis through electrostatic forces¹² can be used to increase the height of liquid rise on the cantilever surface. This approach was retained because as the size of the microstructures becomes smaller their surface wettability can be rather poor even when using hydrophilic surfaces especially with high-aspect ratio narrow features such as microchannels. Moreover, at the microscale, due to the fact that physical scaling laws favor electrostatic forces, effects of electrostatic phenomena on liquid behavior are more pronounced. In addition, associated crippling features such as Joule heating and electrolysis can be drastically reduced on the micrometer scale.

Experimentally, a micropipette is used to deposit a droplet containing the biological sample on a conductive surface. When the end of the microcantilevers is dipped into the droplet, an electric field is applied (10 Volts during 10 seconds) between the passivated aluminum electrodes on the cantilevers and the conductive surface on which the droplet has been deposited. The aim was to apply the low voltage just long enough to have the liquid spread up on the cantilever surface. Thus, both electrolysis and joule heating were minimized. When applying the voltage between the liquid and the passivated electrodes on the cantilevers, electrowetting occurs because the electric field changes the charge distribution on

the surface causing the liquid to spread and wet the surface of the passivated layer thereby filling the microchannels and microreservoirs.

To improve loading efficiency, an AC voltage (10 Volts during 10 seconds at 20 kHz) can be also applied between the two electrodes on the cantilevers immersed in the liquid which is not grounded. In this case, an electromechanical height-of-rise due to electrostatic forces is the main phenomenon.¹³

Whatever the configuration used, these pre-wetted cantilevers are then moved onto the surface where the spots are to be deposited with the three-axis stage with a maximum translation of 200 mm and a positioning precision of 50 nm. All experiments were performed under an ambient condition with a relative humidity of ~30% and a temperature of ~23 °C using a CCD camera to supervise the whole experiment.

In a preliminary experiment, pre-wetted silicon-based microcantilevers have been used to deposit picoliter volumes of a solution containing cyanine3-labelled oligonucleotides (15 mers) on a glass slide. Figure 2 shows a fluorescence image of spots deposited on a glass slide. Spots 30 μm in diameter with a 70 μm center-to-center spacing have been obtained by bringing the cantilevers into very gentle contact with the glass surface. It takes approximately 5 minutes to generate a hundred spot matrix with a single load. During spotting, oligonucleotides are covalently attached to the glass surface by optimized crosslinkers (dendrimer molecules) that react with the amino group on the surface and the fluorescent-labelled oligonucleotide probes. Images were acquired with the Olympus IX70 inverted microscope equipped with a numeric camera. As can be seen in Fig. 2, the shape of the resulting spots is more or less oval due to the fact that they have been obtained without accurately controlling the contact force. Indeed, the deflection of the cantilevers in contact with the surface was controlled optically with the CCD camera. Thus, the deflection could be monitored within the micrometer range leading to an applied force of about 100 nN. Indeed,

with a spring constant of about 0.1 N/m, under increasing the contact force, cantilevers tend to bend or buckle and sometimes slip excessive applied force as can be seen in Fig. 3(a) and 3(b).

As this technique relies on capillary action and diffusion, the spot shape strongly depends on the wettability of the glass surface, time of contact and the angle made by the cantilevers and the surface. This has been evidenced by using both hydrophilic and hydrophobic surfaces. As expected, the spot diameter varies with the square root of the contact time as shown in Fig. 4. This is in agreement with results obtained with the dip pen lithography technique.¹⁴

In a second experiment, using the same procedure, protein microarrays have also been generated on a glass slide coated with dendrimer molecules as crosslinkers. The printed protein is the purified Anti-Goat IgG (rabbit) from Rockland (Ref 605-4102) used in PBS buffer with 40% glycerol. Glycerol was added to prevent evaporation and keep the protein hydrated and activated. The slide was left at room temperature during 3 hours and later immersed in a PBS buffer containing 1% BSA (Bovine Serum Albumin) during 1 hour. This reduces the nonspecific binding of other proteins in subsequent steps on unpatterned glass surface. After a brief wash in PBS buffer, the slide was incubated with the second protein, fluorescein conjugated purified anti-rabbit IgG from Rockland (Ref 611-7202). The interaction between the two proteins was then observed with the inverted microscope. Fig. 5 shows the resulting protein microarray where 30 μm diameter spots were obtained with a contact time of one second.

To fabricate biological microarrays, a crucial feature required for our system is its ability to deposit various biological samples with the same cantilevers without the need to replace them for each sample as it is the case for the dip-pen nanolithography. Here, the strategy was to adapt the conventional cleaning and drying procedures used with a

commercial DNA or protein microspotter. As a consequence, positioning the cantilevers with respect to previously deposited spots on the surface is straightforward since no alignment marks are needed. Moreover, the positioning resolution of the XYZ stage is about 50 nm along the three axis after a mechanical motion to load new biological materials for deposition.¹⁵

Two different biological samples, one containing CY3 fluorescent labels, the other containing CY5 fluorescent labels, have been deposited with the same cantilevers through the optimization of a cleaning, rinsing and drying procedure. The different steps involved in this procedure consist in dipping the cantilevers into pure distilled water and agitating them. These cantilevers were then positioned onto a Peltier dryer at a temperature of 70°C. These steps were repeated two times before loading the second biological sample. 40 spots of each sample were deposited with a single load before cleaning the cantilevers and loading the next biological sample.

As shown in Fig. 6 where the cleaning procedure has been used three times successively, reproducible experiments show no cross contamination between Cy3 and Cy5 solutions. It takes about 10 minutes to deposit in parallel the two matrixes of 4×40 spots, including drying and loading procedures. On the other hand, when the same deposition procedure is performed without cleaning properly the cantilevers, cross contamination of samples could not be avoided.

In conclusion, the results demonstrate that our system could be used to produce high-density DNA and protein chips. Improvements are still needed however with respect to contact force control and cantilever design to reach the submicrometer level and, therefore, the femtoliter range in terms of amount of liquid deposited on the surface. Accurate control within the nN range could be achieved using arrays of these cantilevers with an integrated piezoresistive detection.

It should be added that this approach is fully compatible with a non-contact deposition technique if an electric field is applied between non passivated electrodes along the microchannels on the microcantilevers and a conductive layer. This non-contact method could be improved further to meet the requirements of use as an electrospray technique¹⁶ provided operating conditions allow disintegration of the jet into charged droplets (ionization). Experiments with optimized microcantilevers are underway.

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Figure captions

FIG. 1 : Array of four cantilevers with passivated aluminum electrodes properly designed for electrowetting. The cantilevers are 2 mm long, 210 μm wide, 5 μm thick with a 450 μm spacing.

FIG. 2 : Fluorescence image of spots obtained with a solution containing Cyanine3-labelled oligonucleotides (15 mers) deposited on a glass slide.

FIG. 3 : Optical view of the microcantilevers during the nucleotide deposition sequence. (a) The cantilevers are slightly bent with a low applied contact (under μN). (b) The cantilevers are buckled when applying an excessive applied force.

FIG. 4 : Variation of spot diameter as a function of the square root of the contact time for Cy3-labelled oligonucleotides on a glass slide.

FIG. 5 : Resulting protein microarray after interaction between Anti-Goat IgG (rabbit) and fluorescein conjugated purified anti-rabbit IgG.

FIG. 6 : Deposition of two different solutions with the same cantilevers : (a) Oligonucleotides (15 mers) spots labelled with Cyanine 3 (green) and Cyanine 5 (red) using the cleaning process: no cross-contamination can be observed ; (b) Same experiment without the cleaning procedure: cross-contamination is clearly visible.

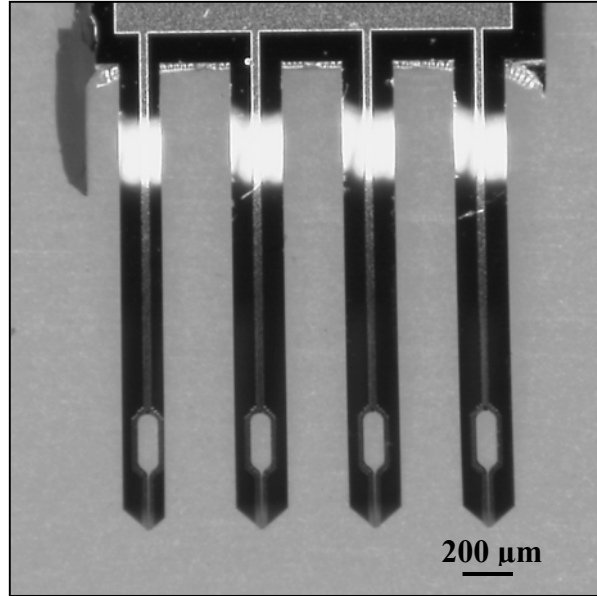


FIG. 1

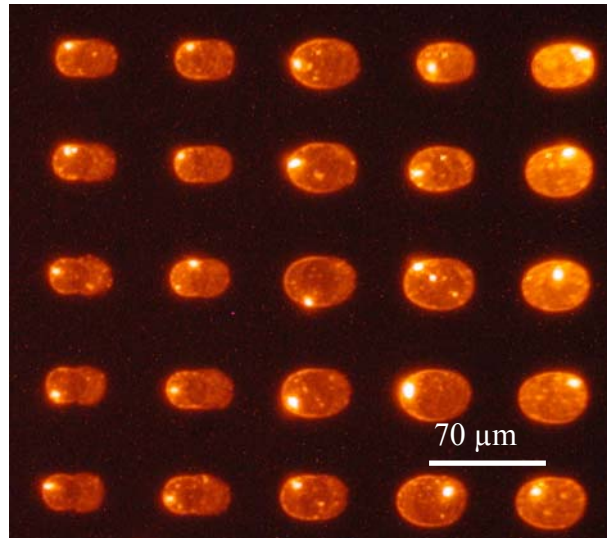
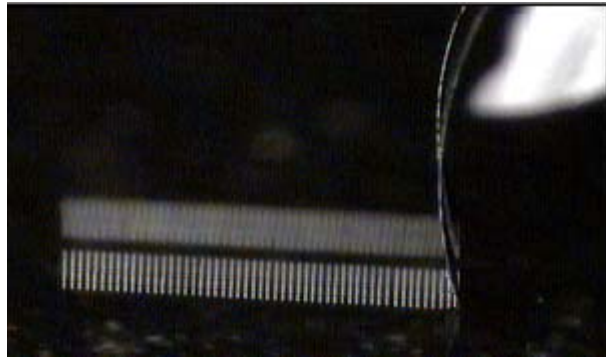


FIG. 2



(a)



(b)

FIG. 3

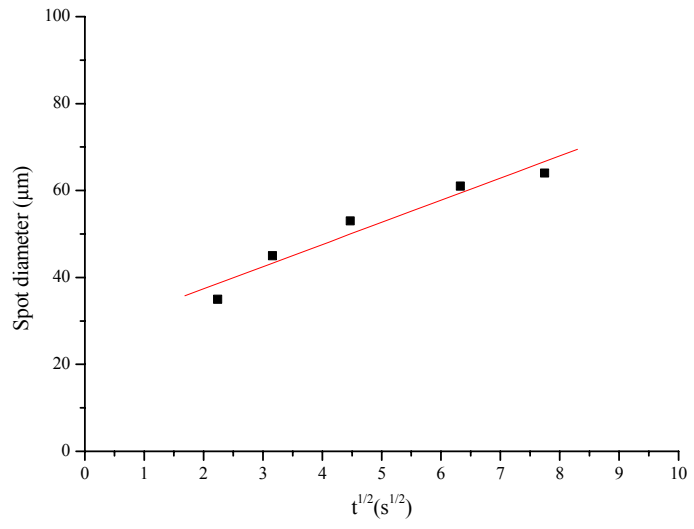


FIG. 4

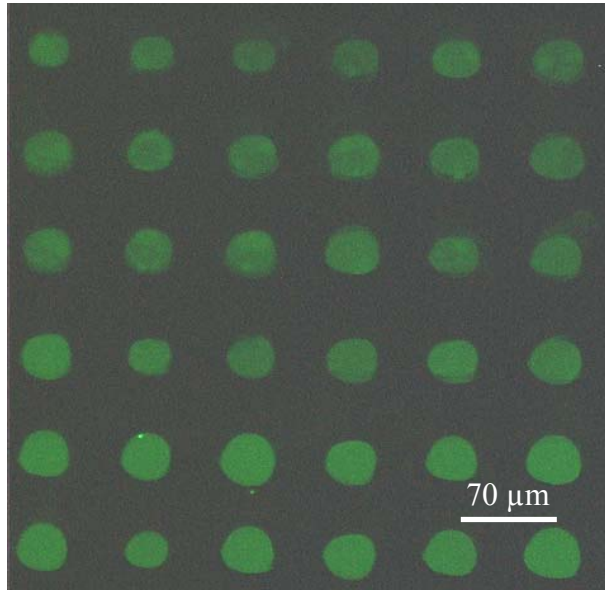


FIG. 5

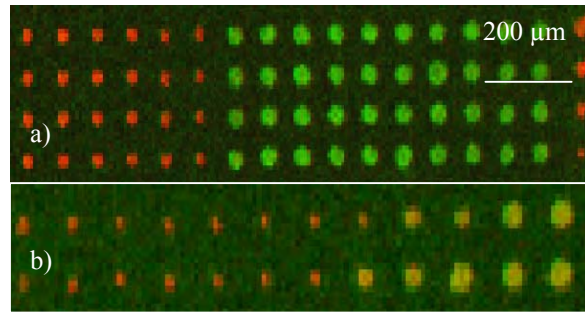


FIG. 6

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